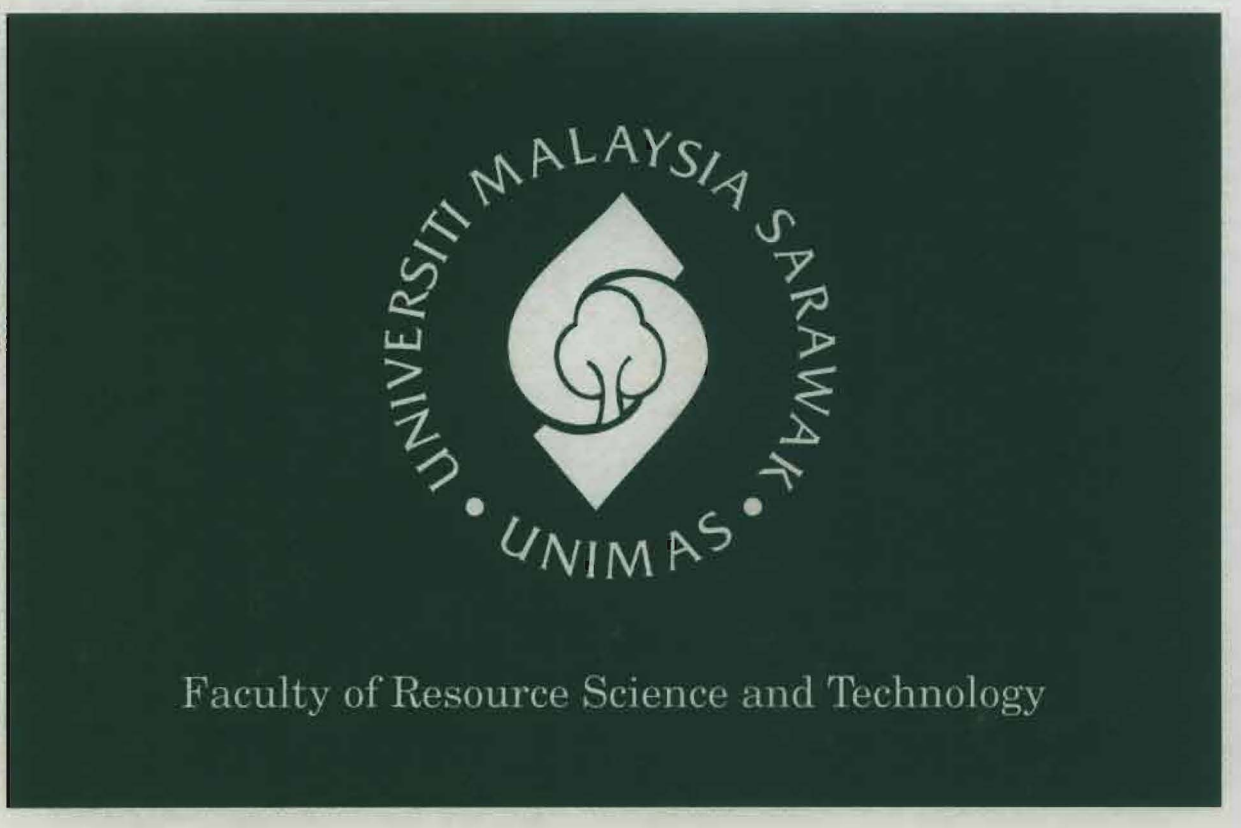

PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON *PIPER SARMENTOSUM*

Kuan Sing Wei

Bachelor of Science with Honours
(Resource Chemistry)
2004

PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON *PIPER SARMENTOSUM*

KUAN SING WEI

This project is submitted in partial fulfilment of the requirements for
the degree of Bachelor of Science with Honours
(Resource Chemistry)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2004

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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ABSTRACT

Phytochemical and biological studies have been carried out on the leaves of *Piper sarmentosum*. The extracts were subjected to thin layer chromatography, column chromatography and preparative thin layer chromatography in order to isolate and purify pure compounds. Two semi pure compounds with the molecular weight of 208 and 270 were isolated. These compounds showed the R_f values of 0.6 and 0.9 in hexane-acetone (7:3). The mass spectra of MA08A and MA09A showed similarity to standard mass spectra of isoasarone. Based on the mass spectra and infra red (IR) information, compound MA09A most probably be isoasarone. In the toxicity test, most of the fractions and crude extracts indicated a low toxicity on the larvae of *Artemia salina*. Only fractions MA03 and MA09 showed LD_{50} at 10 μg/mL, while for fractions MA04 and MA08, the LD_{50} value was 55 μg/mL and 40 μg/mL respectively.

Key words: *Piper sarmentosum*, extraction, fractionation, purification, toxicity test.

ABSTRAK

Kajian fitokimia dan biologi telah dijalankan ke atas daun Piper sarmentosum (Piperaceae). Kaedah kromatografi lapisan nipis, kromatografi turus dan kromatografi lapisan nipis persediaan telah digunakan dalam proses pemisahan dan penulenan. Dua sebatian separa tulen dengan jisim molekul 208 dan 270 berjaya dipisahkan. Sebatian-sebatian ini memberikan nilai R_f 0.6 dan 0.9 dalam sistem pelarut heksana-aseton (7:3). Spektra jisim bagi MA08A dan MA09A menunjukkan kesamaan dengan spektra jisim isoasaron piawai. Berdasarkan maklumat spektra jisim dan infra merah (IR), sebatian MA09A berkemungkinan besar merupakan isoasaron. Ujian ketoksikan ke atas anak udang (larva Artemia salina) menunjukkan kebanyakan fraksi dan ekstrak kasar mempunyai tahap ketoksikan yang rendah. Hanya fraksi MA03 dan MA08 memberikan nilai LD_{50} pada 10 μg/mL, manakala fraksi MA04 dan MA08 masing-masingnya memberikan nilai LD_{50} pada 55 μg/mL dan 40 μg/mL .

Kata kunci: Piper sarmentosum, pengekstrakan, pemfraksian, penulenan, ujian ketoksikan.

INTRODUCTION

The genus *Piper* belongs to the Piperaceae family and has over 700 species distributed throughout the world especially in tropical and subtropical regions and is well known for producing a large number of physiologically active compounds (Parmar *et al.*, 1997). Phytochemical investigation on *Piper* spp. has afforded a diverse range of flavonoids, lignans, neolignans, terpenoids and alkaloids/amides (Jensen *et al.*, 1993; Parmar *et al.*, 1997; Gupta *et al.*, 1999). Some of the compounds isolated from several *Piper* species and their biological activities are given in Table 1. Besides having high commercial and economical importance, *Piper* species are used medicinally in treating various diseases. Some of the *Piper* species studied extensively includes *Piper stylosum, Piper nigrum, Piper hispidum, Piper tuberculatum, Piper aduncum, Piper guineense, Piper methysticum* and many more (Parmar *et al.*, 1997).

Piper stylosum with a local name of "Kadok Hutan" is a sprawling herb with dark green stem and segmented found in the Malay Peninsula, Sumatra and Borneo; in the Peninsula, it is common in forests, almost throughout (Burkill, 1966). The leaves are heart-shape while the flowers and fruits are small in size. Pregnant women use the roots of this species to treat the pain after giving birth or during confinement (Burkill, 1966). Besides that, this species is also used to treat malaria, cough, flu, toothache and rheumatism. The preparations of leaves can be used as medicinal agent for the treatment of hypertension and urinary problems (Mat-Salleh and Latiff, 2002).

Table 1. Some compounds isolated from *Piper* species and their biological activities

Species	Compound(s)	Biological activity	Reference(s)
Piper guineense	$\Delta^{\alpha,\cdots}$-Dihydropiperine	Most potent antifeedant activity	Mensah *et al.*, 1977
Piper retrofractum	(2E,8E)-N-9-(3,4-Methyldioxyphenyl)-nonadienoylpiperidine	Cause dilation of the heart in rabbits	Ahn *et al.*, 1992
Piper sarmentosum	Asaricin α-Asarone γ-Asarone	Inhibitory activities against *E. coli* and *Bacillus subtilis*	Masuda *et al.*, 1991
Piper aduncum	Pseudodillapiole	Strong antimicrobial	Burke and Nair, 1986
	Aduncamide	Antibacterial	Orjala *et al.*, 1993
Piper methysticum	Yangonon	Inhibited the growth of amoeba	Young *et al.*, 1966
Piper betle	Chavibetol acetate	Fungicidal	Grag and Jain, 1996
	Chavicol	Fungicidal	Grag and Jain, 1996
Piper hispidum	N-[7-(3',4'-methylenedioxyphenyl)-2(Z),4(Z)-heptadienoyl]pyrrolidine	Fungicidal	Alècio *et al.*, 1998
Piper tuberculatum	Piplartine	Fungicidal	Homan and Fuchs, 1970
Piper nigrum	Pipericide	Insecticidal	Miyakado *et al.*, 1989

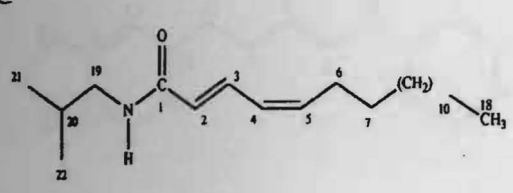
Piper nigrum, which is commonly known as black pepper is externally used as rubefacient and stimulant to the skin and prescribed for cholera, dyspepsia, flatulence, diarrhoea, various gastric ailments and for paralytic and arthritic disorders (Siddiqui *et al.*, 1997; Martins *et al.*, 1998). Amides especially pipericine (**1**) and piperine (**2**) has been isolated from *Piper nigrum* (Siddiqui *et al.*, 1997). *Piper hispidum* and *Piper tuberculatum* accumulate amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties with insecticidal properties (Mikayo *et al.*, 1989; Parmar *et al.*, 1997). Some of the amides such as (3Z,5Z)-*N*-isobutyl-8-(3',4'-methylenedioxyphenyl)-heptadienamide (**3**), *N*-[3-(6'-methoxy-3',4'-methylenedioxyphenyl)-2(Z)-propenoyl]pyrrolidine (**4**) and piperamine (**5**) have been isolated from the stems of *Piper hispidum* while 8(Z)-*N*-(12,13,14-trimethoxycinnamoyl)-Δ^3-pyridin-2-one (**6**), *N*-(12,13,14-trimethoxydihydrocinnamoyl)-Δ^3-pyridin-2-one (**7**), piplartine (**8**), **2**, $\Delta^{\alpha,\beta}$ –dihydropiperine (**9**), 5,6-dihydropiperlonguminine (**10**) and pellitorine (**11**) have been isolated from the seeds of *Piper tuberculatum* (Navickiene *et al.*, 2000). These amides were active against fungus *Cladosporium sphaerospernum* (Homans and Fuchs, 1970).

Piper aduncum is listed as remedies for stomachaches, trachoma and vaginitis (Duke, 1985) due to the presence of benzoic acid derivatives, chromenes and flavonoids with cytotoxic and antibacterial activities (Asprey and Thornton, 1954; Burke and Nair, 1986; Orjala *et al.*, 1993). A chromene, 2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (**12**) and a benzoic acid derivative, 3-(3',7'-dimethyl-2',6'-octadienyl)-4-methoxy-benzoic acid (**13**) have been reported from *Piper aduncum* (Baldoqui *et al.*, 1999). The fruits of *Piper guineense*, West African black pepper have been used as flavorant. Its leaves, roots and seeds have been internally used to treat bronchitis,

gastrointestinal diseases and rheumatism while the extract from the seed kernels have generated interest as a result of their potent insecticidal properties (Irvine, 1961). Kava refers to the rootstock of the plant *Piper methysticum*. The rootstocks and roots may be made fresh into phycoactive beverage. The beverage is not intoxicant, but narcotic (Burkill, 1966). The active ingredients in *Piper methysticum*, kavalactones, have diuretic, soporific, antiepileptic, spasmolytic, analgesic, local anaesthetic, bacteriocidal and antimycotic properties (Lebot and Levesque, 1996).

In this project, *Piper sarmentosum* was studied. *Piper sarmentosum* is used for feverish diseases, for digestive disorders, and toothache (Burkill, 1966). When the root is chewed with betel nut, it is said to be helpful for the treatment of coughs and asthma; with nutmeg and ginger it is used to treat pleurisy. *Piper sarmentosum* is a distinctive species, easily recognized by the creeping, more or less terrestrial habit, virtually unmatched among Asiatic *Piper*. *Piper sarmentosum* has very distinctive leaves, which are usually palmately veined or almost so with a very minute "powdery puberulent" indumentum, and infructescences white at anthesis and with fruits fused to the rachis when mature.

The purpose of this project is to isolate, purify and characterize biological active compounds from *Piper sarmentosum*.



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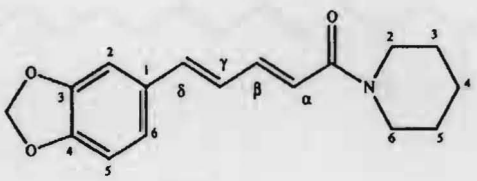

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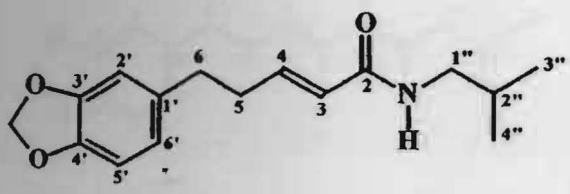

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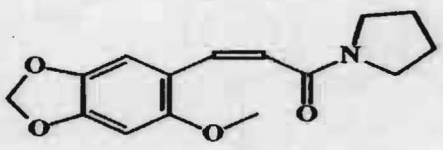

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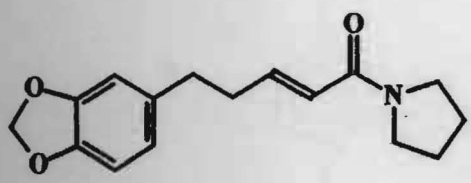

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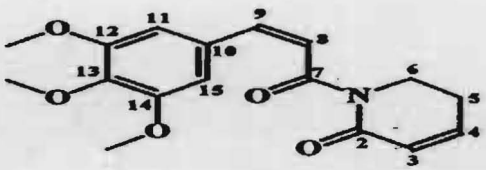

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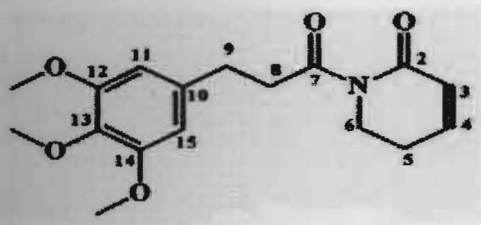

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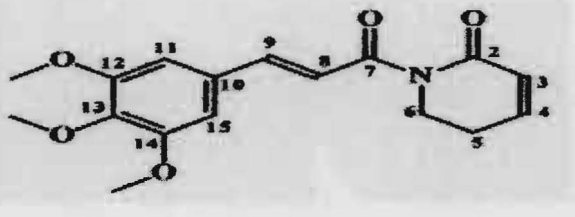

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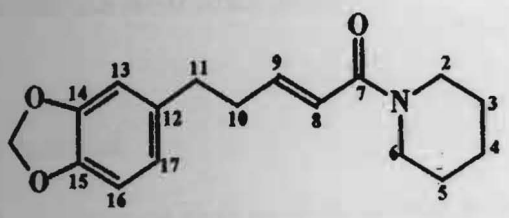

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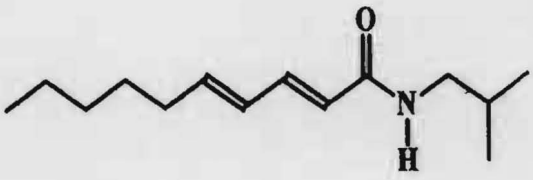

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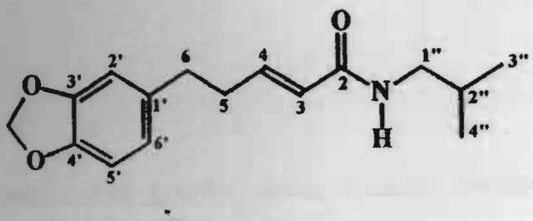

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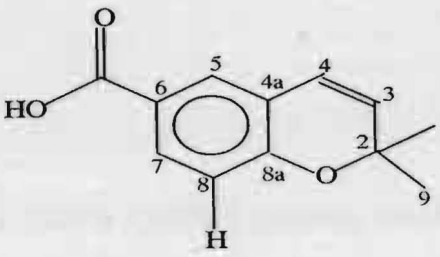

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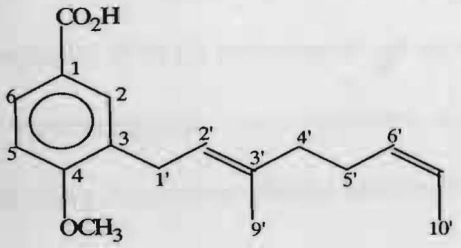

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MATERIALS AND METHODS

Plant Material

Piper sarmentosum samples were collected from the Sarawak Pulp and Paper Acacia Center at Jalan Tabuan, Kuching. Voucher specimen was prepared for the purpose of identification. The samples were air-dried.

General

The sample was grinded using General Electric Model 5KH39 QN5525 grinding machine. Column chromatography (CC) was carried out on silica gel 60 (Merck, 230-400 mesh), thin layer chromatography (TLC) was carried out on silica gel (Merck, Silica gel 60 F_{254}, 0.25mm) and preparative thin layer chromatography (PTLC) was carried out on silica gel (Merck, Silica gel 60 F_{254}, 0.25mm). Spots on the chromatograms were visualized under UV light using UVG-11. The extracts were concentrated using Rotavapor (Buchi Model R-200). The molecular mass of the compounds isolated were determined using Shimadzu QP-5000 Series GS/MS with the capillary column of DB-5. IR spectra were recorded on a Shimadzu FTIR-8201PC spectrometer as a film on a KBr plate.

Extraction, Isolation and Purification

About 1.255 kg of dried and ground leaves of *Piper sarmentosum* was extracted. The dried leaves were extracted with hexane at room temperature for a few days to remove fat. The resulting extract was filtered using gravity filtration. This step was repeated for two times and

all the filtrates were combined and concentrated under reduced pressure using rotavapor. The extraction procedure was repeated using solvents with different polarity such as ethyl acetate and methanol. The extraction using ethyl acetate was repeated for six times and methanol was repeated for three times. Each of the combined filtrate was dried, weighed and the percentage yield was determined.

The concentrated extracts were then subjected to TLC over silica gel plates using suitable solvent. The samples were applied to the plates using a glass capillary. When the spots were dried, the plates were transferred into the developing chamber and were developed using suitable solvent. Retardation factor, R_f value for each spot was determined (Houghton and Raman, 1998). TLC was carried out on ethyl acetate and methanol crude extracts to identify number of chemical compounds present in the extracts. Solvent system of hexane-acetone (7:3) gave the best separation for methanol crude extracts. Six spots were observed on the TLC plates for methanol crude extract. Since the spots on the chromatograms were colourless, they were visualized under UV light or by using spray reagent of 5% sulphuric acid in methanol.

$$R_f = \frac{\text{distance from the point of application of the sample to one of the components}}{\text{distance from the point of application of the sample to the solvent front}}$$

The methanol crude extract was further subjected to CC and eluted with the most suitable solvent system. Column with the size of 60.0 cm in length and 3.0 cm in diameter was used. First of all, the column was checked from two directions to make sure it was vertical. The column was rinsed by pouring acetone and the suitable solvent system. Then, by using a glass rod, cotton of about 1 cm thick was pushed to the bottom of the column to avoid the silica gel from being eluted. With the stopcock closed, about 20 mL of solvent system was drained from the column to ensure that all the air bubbles have been displaced. About 350 mL of hexane-acetone (7:3) was used to dilute the silica gel. Then, the column was filled with the silica gel slurry that has been made with suitable solvent.

About 5.0 g of sample was added into the column and eluted using solvent with increasing polarity. About 25 mL eluent was collected for each fraction. These fractions were subjected to TLC and the fractions with similar R_f value were combined, dried and the weight of combined fractions was recorded. PTLC using hexane-acetone (7:3) was carried out to purify the combined fractions. After development, the compounds were scrapped out from the plates and dissolved in similar solvent used for PTLC fractionation. All the samples were filtered and dried.

Structural Determination

The purity of the sample isolated and the mass spectra was determined using GS/MS. The initial temperature for GC/MS was 50°C and increased to 320°C with the rate of 6.5°C/minute. The final temperature was maintained for 10 minutes. Before injecting the sample, dichloromethane

(DCM) was injected into the capillary column in order to clean the column from impurities. The sample was diluted in DCM before injected into the GC/MS.

Functional Groups Determination

The sample for FTIR was prepared according to standard techniques. About 1.0 mg sample was ground with 100.0 mg of potassium bromide (KBr). The mixture was compressed in the form of tablets of about 1 mm thick under pressure. FTIR spectra of sample were recorded in the range of 400 cm^{-1} – 4000 cm^{-1} using Shimadzu FTIR-8201PC spectrometer.

Brine Shrimp Toxicity Test

Toxicity assay on the crude extracts and fractions were carried out using brine shrimp (*Artemia salina)* toxicity test according to the method established by McLaughlin (1991). About 2.0 g of the shrimp eggs were transferred into 1 L of seawater (salinity 22.0 ppt, temperature 28.0°C and pH 7.5) in order to hatch the eggs. Continuous air was provided during the hatching process. Extract (2.0 mg) was dissolved in 2.0 mL of methanol. From these solutions, 5 µL, 50 µL and 500 µL of samples were transferred to each vial in three replicates. The solvent from the extract was removed using rotavapor and 5.0 mL of seawater was added to each vial, resulting in final concentrations of 1 µg/mL, 10 µg/mL and 100 µg/mL. Then, 2.0 mL of sample was transferred to the *NUNC* multidish and second instar larvae of *Artemia salina* (10 per vial) were added. The survivors were counted after 24 hours contact and the LD_{50} was calculated. Controlled test was carried out with the same method using seawater.

RESULTS AND DISCUSSION

Extraction, Fractionation and Purification of *Piper sarmentosum*

Ground leaves of *Piper sarmentosum* were extracted for several days at room temperature with hexane. The extraction using hexane was repeated twice in order to remove fat or fatty acid component in the plant. The residue was further extracted with ethyl acetate for six times until the dark green colour of the plant faded in order to extract less polar compounds such as terpenoids especially sesquiterpenoids. The final solvent used in extraction was methanol and this procedure was repeated twice to extract polar compounds such as flavonoids, alkaloids, xanthon and quinones. The crude extracts were evaporated to dryness under reduced pressure. The weight obtained for each crude extract is given in Table 2.

Table 2. Weight and percentage yield from the extraction of *Piper sarmentosum* leaves

Solvent	Weight (g)	Percentage yield (%)
Hexane	15.13	1.21
Ethyl acetate	312.47	24.90
Methanol	128.15	10.21

Ethyl acetate and methanol crude extracts were subjected to TLC analysis using suitable solvent. Methanol crude extract was further fractionated and purified to isolate and characterize polar compounds such as flavonoids, alkaloids, xanthon and quinones.

Methanol crude extract was subjected to TLC with the solvent system of hexane-acetone (7:3). Six spots were observed on the TLC plate under UV light (Appendix 1). The R_f values for each spots are shown in Table 3. Ethyl acetate crude extract gave four spots on the TLC plate using solvent system of hexane-acetone (9:4)(Appendix 2) and the R_f values are shown in Table 4.

Table 3. R_f values for components of the methanol crude extract of *Piper sarmentosum* in the solvent system of hexane-acetone (7:3)

Component	R_f Value
1	0.24
2	0.34
3	0.44
4	0.52
5	0.58
6	0.62

Table 4. R_f values for components of the ethyl acetate crude extract of *Piper sarmentosum* in the solvent system of hexane-acetone (9:4)

Component	R_f Value
1	0.54
2	0.62
3	0.70
4	0.76

The methanol extract was subjected to silica gel CC and eluted with five solvent systems with increasing polarity, which was hexane-acetone (7:3), (5:3), (1:1), acetone and methanol. This fractionation procedure afforded 88 fractions with fractions 1-60 from hexane-acetone (7:3), fractions 61 and 62 from hexane-acetone (5:3), fractions 63-65 from hexane-acetone (1:1), fractions 66-68 from acetone and fractions 69-88 from methanol. All the fractions were subjected to TLC analysis. Fractions with similar R_f values were combined and a total of 20 combined fractions were obtained. Table 5 gives the weight for each combined fraction obtained from CC.

Combined fractions of MA03, MA08 and MA09 were subjected to PTLC using hexane-acetone (7:3). These fractions were chosen because they gave the best separation on TLC. After development, seven bands on PTLC from fractions MA03, MA08 and MA09 were scrapped out from the plates. The major compound from each PTLC plate was dissolved in hexane-acetone (7:3). All the samples were filtered and dried. The R_f value for MA03A was 0.9 while fractions MA08A and MA09A showed R_f value of 0.6 in hexane-acetone (7:3) (Appendix 3-5). Although one spot was observed under UV light on the TLC plates from the fractions respectively, the compounds were not truly pure as indicated by GC analysis.

Table 5. Weight of the combined fractions obtained from column chromatography on methanol crude extract from *Piper sarmentosum*

Combined fractions	Fractions	Weight (mg)
MA01	1-5	4.6
MA02	6	5.0
MA03	7-8	51.7
MA04	9-10	37.9
MA05	11	24.6
MA06	12-14	31.3
MA07	15-18	35.9
MA08	19-27	30.5
MA09	28-32	168.6
MA10	33-36	145.1
MA11	37-41	78.6
MA12	42-45	81.8
MA13	46-55	120.0
MA14	56-64	39.3
MA15	65-69	133.8
MB16	70-72	80.9
MB17	73-76	98.1
MB18	77-80	100.3
MB19	81-85	106.5
MB20	86-88	77.6

Notes: Fractions MA01-MA15 were developed in hexane-acetone (7:3); while fractions MB16-MB20 were developed in ethyl acetate-methanol (1:1).

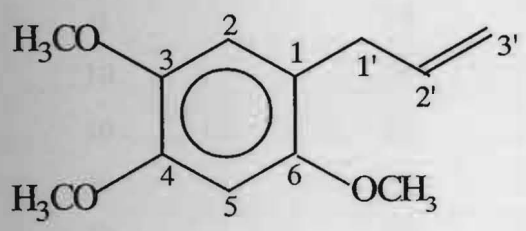
Structural Determination of the Isolated Compounds

MA03A, MA08A and MA09A were subjected to GC/MS analysis to determine the purity and the molecular weight of the compounds. About 1.0 mg of sample was dissolved in 200 µL DCM. MA03A showed one sharp peak at retention time 27.017 min and several other peaks (Appendix 6). Based on the mass spectrum, the compound MA03A has the molecular mass (m/z) of 270 (Appendix 7).

MA08A and MA09A showed one sharp peak at retention time 23.017 min and 23.000 min respectively (Appendix 8 and 10). Compound MA08A and MA09A both showed the m/z of 208 (Appendix 9 and 11). The m/z (relative intensities) for MA09A is 208 [m^+] (100), 193 (53), 177 (7) and 164 (42). The m/z value and fragmentation pattern for MA09A are similar to the published information (Santos *et al.*, 1998).

MA09A was subjected to FTIR to determine the functional groups present in the compound. The IR spectrum (Appendix 12) revealed an absorption band at frequency 1510.2 cm^{-1} with high intensity that was ascribed to a C=C conjugated bond in aromatic compound. The absorption at frequencies 1201.6 cm^{-1}, 1101.3 cm^{-1} and 1001.0 cm^{-1} indicated the presence of C–O bond. These three absorptions displayed a high possibility to be ester compound. The absorption at frequency 2840.9cm^{-1} indicated the presence of C-H bond (stretching) in the compound. The absorption at frequency 1401.9 cm^{-1} indicated the presence of $-CH_3$ bond of alkane compound.

ased on this comparison, MA09A most probably be isoasorone (**14**). Isoasarone has been olated previously from *Piper marginatum* (Santos *et al.*, 1998). Isoasarone has not been ported from *Piper sarmentosum*. Previous studies on *Piper sarmentosum* have reported the resence of asaricin (Masuda *et al.*, 1991).



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Brine Shrimp Toxicity Test

Brine shrimp toxicity test was carried out on the ethyl acetate crude extract, methanol crude extract and combined fractions (Appendix 13). Based on the toxicity results, fractions MA03 and MA09 showed the LD_{50} at the concentration of 10 µg/mL respectively while for fractions MA04 and MA08, the LD_{50} values were 55 µg/mL and 40 µg/mL respectively. However, the remaining samples showed a low level of toxicity with LD_{50} value exceeding 100 µg/mL as indicated in Table 6.

ble 6. Average death of *Artemia salina* (%) as a function of concentration for methanol crude extracts and fractions from *Piper sarmentosum*

mple	Average of Artemia salina death (%) as a function of concentration		
	1 µg/mL	10 µg/mL	100 µg/mL
OH crude extract	0	10	20
A01	10	20	30
A02	10	20	40
A03	30	50	60
A04	30	40	60
A05	0	10	40
A06	10	20	40
A07	10	20	40
A08	30	40	70
A09	10	50	70
A10	10	20	40